COUNTER TERRORISM MEASURES TO COMBAT YERSINIA PESTIS WITH SELENIUM PHARMACEUTICALS

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ABSTRACT

The purpose of this study is to produce selenium labeled peptides and phage (bacterial viruses) that can selectively bind to the surface of the pathogenic bacteria Yersinia pestis (the plague) and inactivate it through the generation of superoxide radicals on its surface, similar to the way that it is inactivated by superoxide in macrophage. The results show that phage specific for a given bacteria can be obtained from phage expression libraries. These same phage can be covalently labeled with selenium and still retain their binding ability. Selenium labeled phage can kill targeted bacteria that are resistant to the phage without selenium.

INTRODUCTION

The plague or Black Death is an infection of rodents caused by *Yersinia pestis* and can be transmitted to humans by the bite of infected fleas. The disease is manifested in bubonic and pneumonic forms. Within hours of the initial fleabite, the infection spills out into the bloodstream, leading to involvement of the liver, spleen, and lungs. The patient develops a severe bacterial pneumonia, exhaling large numbers of viable organisms into the air during coughing fits. Fifty to sixty percent of patients will die if untreated. As the epidemic of bubonic plague develops (especially under conditions of severe overcrowding,), it eventually shifts into a predominately pneumonic form, which is far more difficult to control and which has 100 percent mortality. For obvious reasons, this disease has military importance. In order to develop a selective antibiotic to the disease that would only target these bacteria we have focused on the F1 antigen that is found on the surface of the bacteria.

The capsular antigen fraction 1 (F1) is a protein-polysaccharide complex, presumably synthesized as a glycoprotein (1,2), that forms a major component of the outer membrane capsule of Yersinia pestis (3-5). Y. pestis exhibits diminished capsular expression at normal room temperature, but switches to full expression when grown at 37^{o}C (3,6). Capsular production also confers resistance to phagocytosis (6). Direct evidence that F1 can function as a protective immunogen was originally reported by Baker et al. (7) and was confirmed by Zakrevskiy and Plekhanova (8). Recently, as little as 1 ug of recombinant F1 cut from an acrylamide gel protected mice against 10^5 parenterally administered virulent organisms (9). Furthermore, mice immunized with live recombinant Salmonella strains expressing F1 were protected against a parenteral plague challenge (10). Thus, F1 expression is required by Y. pestis in the body and is a good target site for an antibiotic.

In order to make a targeted antibiotic toxic we have chosen to use the element selenium. The paradox of selenium is that in the rat it is essential in the diet at 0.20 ppm but is highly toxic at 16 ppm

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(11). An average adult human requires approximately 20 ug Se/day for normal function (while the RDA is 55 ug/day). Dietary selenium from the inorganic salts, selenate and selenite, and the organic selenium compounds, mainly the primary protein amino acid selenomethionine, are metabolized by the body into selenocysteine, which is found in selenium enzymes and proteins fulfilling the dietary requirement for selenium (12).

Very recently, an understanding of selenium as a catalytic generator of superoxide radicals ($O_2^{-\cdot}$) from the oxidation of thiols has emerged. This catalytic attribute of selenium has been known for nearly five decades, but the pro-oxidative characteristics of several selenium compounds has only recently been elucidated. In general, selenite and selenium dioxide are catalytic and produce superoxide by their interaction with thiols, such as glutathione (GSH), forming the glutathione selenide anion, $GSSe^-$. Diselenides, such as selenocystine, are reduced by thiols forming the selenide anion, RSe^-, which is catalytic. The selenide anion is the catalytic species, catalyzing the further oxidation of thiols producing superoxide radicals, hydrogen peroxide (H_2O_2) and a putative thiyl radical. These and likely other reactive oxygen species appear to account for selenium's toxicity. The selenide anion in these small organoselenium compounds which produces $O_2^{-\cdot}$, is also the catalytic moiety of the selenocysteine residue in all known selenium enzymes (11).

$$\mathbf{R\text{-}Se^- + O_2 \rightarrow R\text{-}Se^{\cdot} + O_2^{-\cdot}}$$
$$\mathbf{R\text{-}Se^{\cdot} + 2\ R^*\text{-}SH \rightarrow R\text{-}Se^- + R^*\text{-}S\text{-}S\text{-}R^*}$$

The above mechanism is why selenium is toxic. As can be seen, $R\text{-}Se^-$ can react with oxygen to form a superoxide radical and a selenide radical. The selenide radical can then be reduced back to $R\text{-}Se^-$ by a thiol such as glutathione.

We have found that attachment on the surface of a cell is required for selenium toxicity. If selenium is attached to an antibody specific for a human red blood cell, it can lyse the cell due to the generation of superoxide radicals on the surface of the cell (13). However, if the cell is pretreated with native antibody that does not contain selenium before being exposed to the selenium-antibody complex, no cellular lysis occurs. This is because superoxide produced in solution is diluted by the media and its diffusion lifetime is too short to cause lysis. Human cells have enzyme mechanisms to quench superoxide radicals (i.e. superoxide dismutase). However, if the dose of superoxide is concentrated enough in a localized area you can overwhelm a cell and lyse it. Thus, superoxide radicals only cause cell damage if they are produced in a localized concentration on the surface of a cell.

In other experiments we find that organo-selenium compounds covalently attached to an insoluble matrix are effective in blocking bacteria from binding to the matrix and in killing bacteria on the surface of the matrix (14).

The present study was carried out to assess the ability of selenium labeled phage and selenium labeled peptides to target and inactivate Y. pestis. The premise of the study is that using phage expression libraries we should be able to obtain peptides that specifically bind to the F1 antigen of Y. pestis. Then using these peptide sequences we should be able to covalently attach selenium to the phage or peptide yielding an antibiotic that targets and kills the bacteria.

EXPERIMENTAL METHODS

BIOPANNING WITH THE PHAGE PEPTIDE LIBRARY

To find the common amino acid sequences of the phage that will bind to the F1 antigen with high affinity, we carried out three rounds bio-panning of a Ph.D 12 library (New England Biolabs Inc.) to the

F1 antigen attached to a plastic dish. After the third round of bio panning, we randomly chose different phage and amplified the phage separately. We then tested for the binding affinity of this phage to bacteria expressing the F1 antigen. We incubated each phage with E. coli expressing the F1 antigen (10^{11} phages per 10^{8} Bacteria) in a 1.5ml microcentrifuge tube at room temperature for 10 minutes. The bacterial cells were spun at 15,000 rpm for 2 minutes. We transferred the supernatants to separate tubes, and titered the unbound phage by a plaque assay. Also, bound phages were eluted from the bacteria with 1ml of glycine elution buffer (pH. 2.2). After 10 minutes of elution time, the unbound phage were removed by centrifugation and then neutralized with 150ul of Tris-HCl (pH. 9.1) and tittered. We found that 90% of the phage bound with a ratio of 1000 phages per bacteria. The phage were then sent for DNA sequencing.

TESTING OF PHAGE ON F1 ANTIGEN EXPRESSED ON THE SURFACE OF E. COLI

Using an F1 construct supplied by Tom Schwan at the Rocky Mountain Laboratories in Hamilton, MT we were able to show that the F1 antigen from Y. pestis could be expressed in E. coli (PYPR-1b) and that the protein is found on the surface of the cells. Phage selected for binding to the F1 antigen were shown to preferentially bind to the bacteria that expressed the F1 protein (PYPR-1b) over the wild type strain (XLI-Blue), which does not express the F1 protein. These experiments were carried out by the following protocol: The bacterial cells are grown to approximately $1x10^{8}$ bacteria/ml of culture. One ml of bacterial culture is placed in a 1.5ml microcentrifuge tube and the cells were then spun down and the supernatant was discarded. The cell pellet is resuspended in 1ml TBS (pH 7.4), vortexed, spun down, and the supernatant solution discarded. The cell pellet is then resuspended in 300ul of phage that was pretreated with an antibody to the phage ($1x10^{11}$ Phage and 1:10,000 dilution of HRP conjugated anti-M13 antibody in 300ul TBST 0.5%). This is allowed to sit on ice for 1 hour. After incubation on ice, the cells are spun down, resuspended in 300ul of TBST 0.5%, vortexed, and spun down again. This washing step is repeated five times. After the last spin, the cell pellet is resuspended in 100ul of TMB substrate. This was allowed to sit at room temperature for 10 minutes and then 100ul TMB stop solution is added. The cells are then spun down and 100ul of the supernatant is transferred to microwell plate and read at 450nm by a microplate reader.

LABELING OF PHAGE WITH SELENIUM

Filamentous coliphage M13 used for the phage expression libraries express a huge excess of coat protein relative to the minor coat protein (pIII) used for binding, these were used for the covalent attachment of organo-selenium. This synthesis was achieved by using an α-cyanoseleno-acetic acid. The acid group of the organo-selenium was activated with dicyclohexyl-carbodiimide followed by the addition of N-hydroxy-succinimide. This activated material was then allowed to react with the amino groups on the coat proteins of the phage. The phage were then spun down and washed 5 times to remove any un-reacted selenium. The phage were then tested for the attachment of selenium by chemical analysis of selenium content.

RESULTS

USE OF PHAGE-DISPLAY TO ISOLATE AND IDENTIFY HIGH-AFFINITY RECEPTOR LIGANDS, WHICH CAN BIND SPECIFICALLY TO THE F1 CAPSID PROTEIN OF Y. PESTIS

The 18 sequences below were selected from 60 different sequences found from bio-panning with phage expression libraries for peptide sequences that would bind preferentially to the purified F1 antigen. This library contains 1.9×10^{9} independent clones.

#1	SFSLKPHASLIR	#11	GWFSTPLKWRMQ
#2	GPNKFSLMHLFS	#12	SNFTLPFLKTFR
#3	SFSLSSYSALLW	#13	SWFTLHNLPNRP
#4	KFSLSPHTAWFL	#14	NFSINPRMMWPV
#5	KLSLNPHFMFQS	#21	FSIKHPWPFFLP
#6	FSLKNPTIANTM	#28	FSIKLPYWQRTF
#7	LISVEPASLSAH		
#8	SSLTLAPFSWSL		
#9	GPWFSLRHLSPQ		

To find the common amino acid sequences of the phage that bound to the F1 antigen with high affinity, we carried out the three rounds bio-panning of the Ph.D. 12 library on the antigen attached to a plastic dish. After the third round of bio-panning, we randomly chose different phage clones and amplified those phage separately. We then sequenced the phage DNA from the clones. From the DNA sequences, we found 60 different sequences. As can be seen from the above, although over 1.9×10^9 independent sequences were screened, common sequences appears in the 15 isolates. One rather long sequence is the sequence Phe-Ser-Leu-Lys that appears in isolates #1 and #6. Additionally a portion of this sequence [Phe-Ser-Leu] appears in four other sequences (#2, #3, #4, #9) and a similar sequences [Phe-Thr-Leu] appears in #12 and #13, and Phe-Ser-Ile in #14, #21 and #28. An additional Ser-Leu sequence appears in sequences #1, #7, and #8. Thus, the Phe-Ser-Leu, Phe-Thr-Leu and Phe-Ser-Ileu motifs appears in 11 out of 15 sequences. In addition, In many cases (11/15) a proline appears in the center of the motif and a lysine appears next to the FSL(I) motif (6/15).

LABELLING OF THE PHAGE WITH SELENIUM

We found that we could attach up to 300 selenium atoms per phage without affecting the ability of the phage to bind to a bacteria. W e then tested the phage for the ability of the selenium attached to the phage to inactivate the phage from the standpoint of its ability to infect bacteria. The following experiments were carried out to test the hypothesis that we can label a phage with selenium and it can then kill bacteria for which it is not cytotoxic. This is shown below in Figure 1. bind a large amount of organo-selenium without effecting the binding ability of the phage. These phage then serve as targeting vehicles to bind selenium to the surface of Yersinia pestis

As can be seen from Figure.1, the selenium labeled phage loses its ability to infect bacteria with time. As will be seen in later results, this does not impair its ability to bind to the bacteria and kill the bacteria. It can kill because selenium does not have to be internalized to kill and has nothing to do with the infection process. The phage is only needed as a specific targeting vehicle for the selenium.

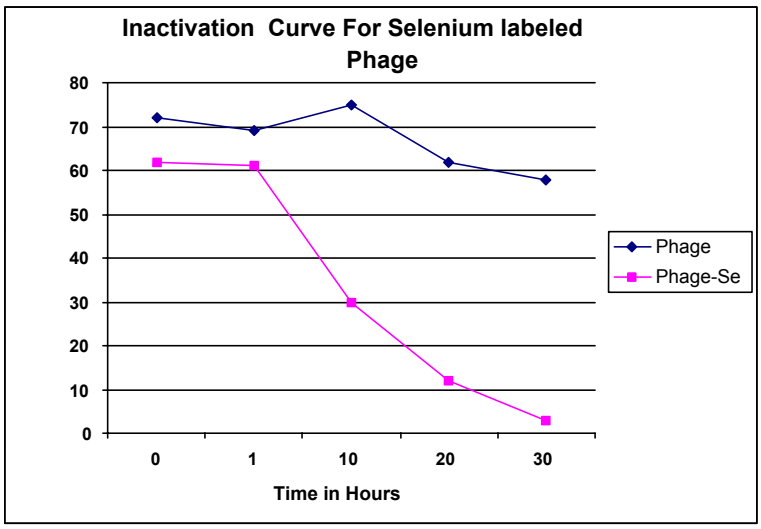

Figure 1. Inactivation Curve for Selenium labeled Phage.

Figure 2, below, shows that all of the F1 binding selected phage show a preference for the bacteria that expresses the F1 antigen, over the wild type bacteria. We found that at lower levels of phage to bacteria that an even greater preference is obtained. PC1 and PC2 were phage selected by a different process and did not show the desired specificity.

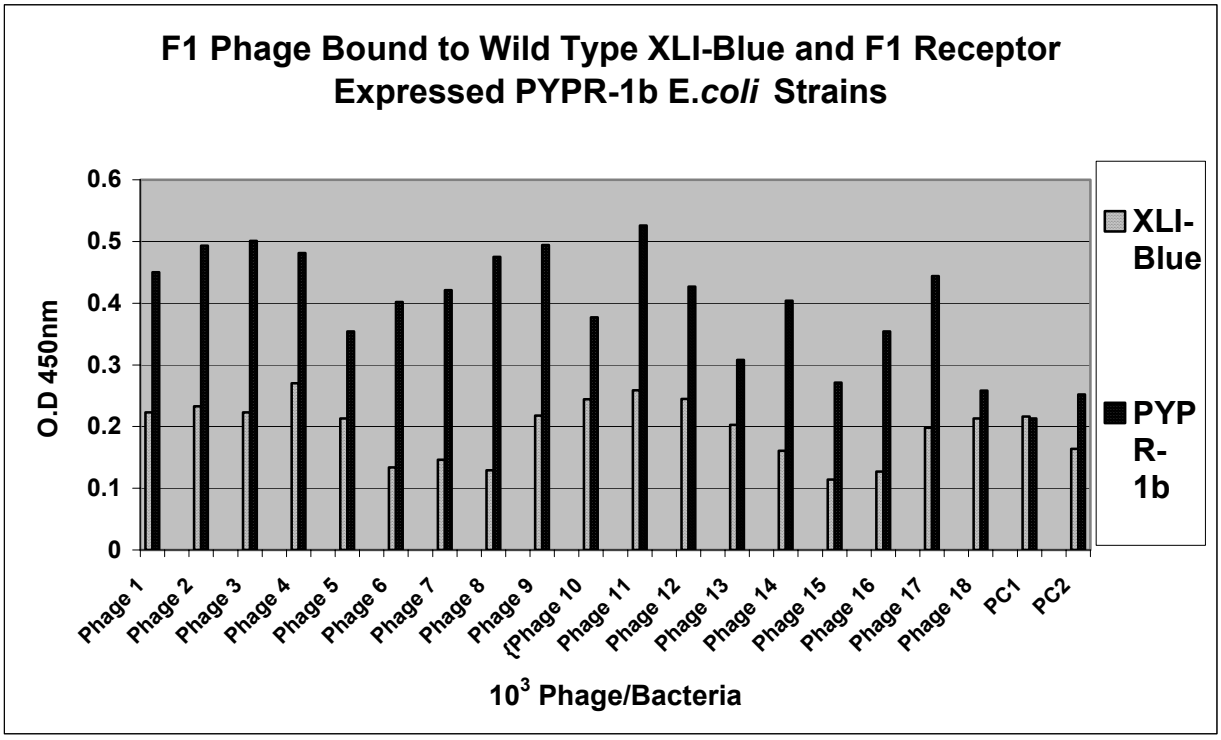

Figure 2. F1 Phage Bound to Wild Type XLI-Blue and F1 Receptor Expressed PYPR-1b E. coli Strains.

Figure 3 below shows that the phage can kill the bacteria. It kills better in the presence of glutathione (there is approximately 150 uM glutathione in human blood). It can also be seen that there is an immediate killing and then a slower killing.

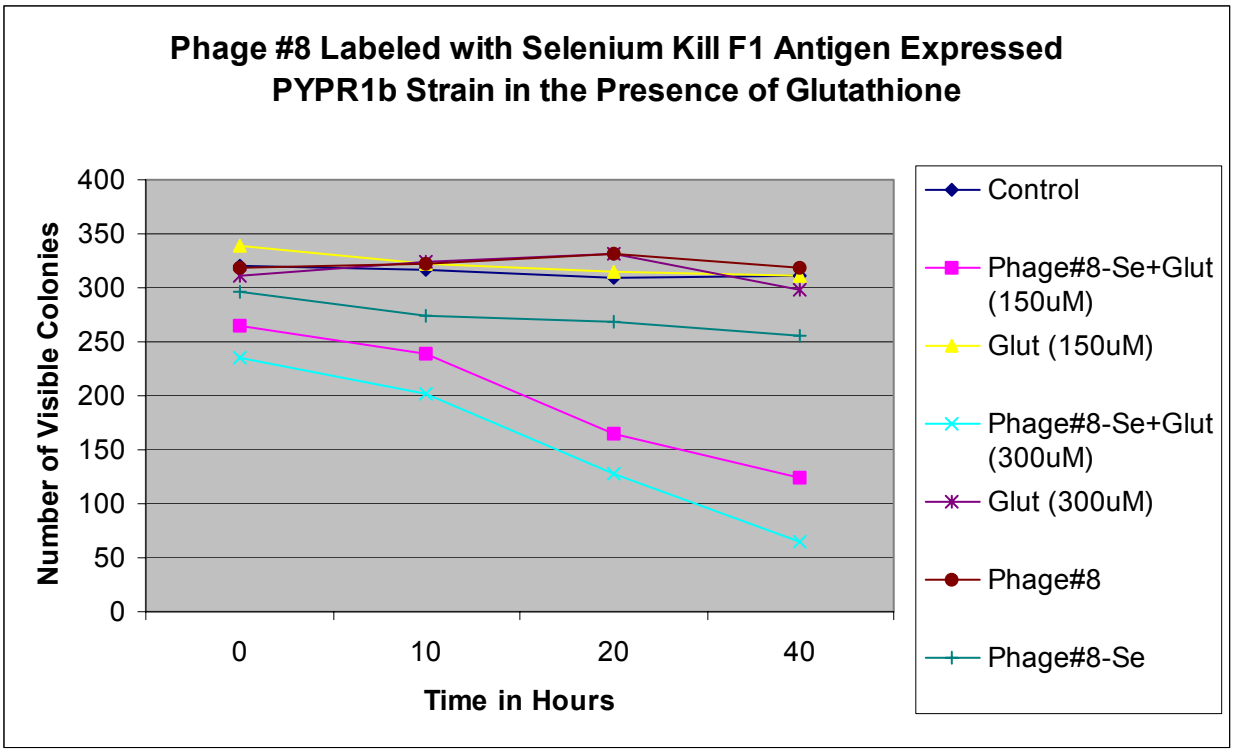

Figure 3. Phage #8 Labeled with Selenium Kill F1 Antigen Expressed PYPR1b Strain in the Presence of Glutathione.

In additional experiments we showed that phage #8 (Figure 3) could compete with antibody to the F1 antigen. Thus, not only can it kill the bacteria expressing the F1 antigen, but it binds quite tightly.

CONCLUSIONS

- We can isolate phage that bind specifically and tightly to the F1 antigen.
- We can covalently attach organo-selenium molecules to these phage without affecting their
- binding ability to the bacteria.
- We can show that these organo-selenium labeled phage can kill the targeted bacteria.
- The killing mechanism is dependent upon the presence of a source of external electrons such as
- glutathione.
- Based upon the sequences determined by the phage expression libraries for binding to the F1 antigen, we can now synthesize peptides that contain these same sequences which would allow us to treat with higher concentrations.

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